SOUTH AFRICAN TB VACCINE INITIATIVE
(SATVI)
CLINICAL TRIALS LABORATORY

**Title:**
**Analysis plan for the primary endpoint of MTBVAC 201.**

Protocol Title: A randomized, double-blind, dose-escalation clinical trial of the safety, reactogenicity and immunogenicity of MTBVAC compared to BCG Vaccine SSI, in newborns living in a tuberculosis endemic region with a safety arm in adults. Final Version 03. Dated 19 February 2015.

**Compiled by:**

Name: Dr Helen Mearns

Position:

Sign: _____

Date: 4 December 2017

**Authorised by**:

Name: Assoc Prof. Thomas Scriba

Position: Deputy Director Immunology

Sign: _____

Date: 4 December 2017

## Background and protocol summary

This document describes the primary endpoint immunology analysis to be performed on the immunogenicity data obtained from the MTBVAC Phase 1 clinical trial conducted in neonates. This plan is to be read in conjunction with the approved study protocol version 03.

Management of the immunology data will be the responsibility of the designated laboratory technologists and the Postdoctoral Scientists under the supervision of the Deputy Director of Immunology, SATVI and the study PI.

## Statistical analysis

Statistical analyses of immunology will be performed using statistical software including Prism (GraphPad Software Inc.) and R. Where significance is reported, the 5% level of significance will be used and adjustments for multiple comparisons using the Benjamini-Hochberg false discovery rate.

## Immunogenicity analysis

The immunology analyses will be conducted on all randomized participants who received a vaccination.

## Trial Objectives

Primary objectives:

1. To evaluate safety and reactogenicity of MTBVAC, compared to BCG Vaccine SSI, in healthy, BCG-naïve, HIV unexposed, South African newborn infants.
2. To evaluate immunogenicity of MTBVAC, compared to BCG Vaccine SSI, in healthy, BCG naïve, HIV unexposed, South African newborn infants.

Secondary objectives:

1. To evaluate safety and reactogenicity of MTBVAC, compared to BCG Vaccine SSI, in healthy, BCG vaccinated, HIV negative, QFT negative, South African adults.

**Trial Endpoints**

Immunogenicity endpoints (in infants only): Primary immunogenicity endpoint

1. Frequencies and co-expression patterns of CD4 and CD8 T cells producing IFN-γ, TNF-α, IL-2 and/or IL-17 induced by MTBVAC or BCG, and suitable antigens in healthy, BCG naïve, HIV unexposed, South African newborn infants.

Exploratory immunogenicity endpoints

1. Whole blood supernatants samples for further immunogenicity tests will be collected and stored frozen. Exploratory assays will be planned, based on data from the primary immunogenicity analyses.

**Primary Immunogenicity Objectives**

1. To determine whether the peak immune response following vaccination is greater than the respective immune response at baseline.
2. To determine whether the immune response at the end of study duration is greater than the respective immune response at baseline.
3. To determine whether the magnitude of the immune response is dose dependent.
4. To determine whether vaccination drives antigen specific CD4 and CD8 T cells.
5. To determine the character of the vaccine-induced response in terms of T helper function (Th1, Th17, etc) and cytokine co-expression (monofunctional vs polyfunctional, etc).

**General definitions for analysis**

**Study period**

Analysis of primary immunogenicity data commences from the time of performing the first analysis to the last completed analysis. Data are reported from blood samples taken at pre-defined study visits from individual study participants and analyzed retrospectively (Table 1).

***Study groups***

Cohorts 1-3 will include 36 participants. Study participants will be randomized to receive MTBVAC or BCG as outlined in Table 2 below. Immunogenicity analyses will be reported per cohort to enable analysis of dose response. BCG recipients will be pooled into a single group.

**Table 1: Summary of Immunology Laboratory Evaluations:**

| Sample Type | Assay | Immunology Endpoint | Approximate Blood Volume (per Visit) | Study Days | Name and Location of Analysis Laboratory |
|---|---|---|---|---|---|
| Whole blood | 12-hour WB-ICS[a] for analysis of T cell responses | Primary and Secondary | 0.75 mL or 2.5 mL | 7, 28, 70, 180 | SATVI, South Africa |
| Supernatants from whole blood ICS assay | ELISA/ Multiplex bead array for analysis of innate response cytokines | Exploratory | NA | 7, 28, 70, 180 | SATVI, South Africa |

NA: not applicable.

a. Stimulation conditions: MTBVAC and a pool of 122 Mycobacterium immunodominant epitopes plus controls, Nil and PHA.

**Table 2: Treatment Groups and Number of Participants:**

| Cohort[a] | Number of Doses | Treatment Assignment | | | Total N |
|---|---|---|---|---|---|
| | | MTBVAC[b] | | BCG ($5 \times 10^5$ CFU)[b] | |
| | | Dose Level | N[c] | N | |
| 1 | 1 (Day 0) | $5 \times 10^3$ CFU | 9 | 3 | 12 |
| 2 | 1 (Day 0) | $5 \times 10^4$ CFU | 9 | 3 | 12 |
| 3 | 1 (Day 0) | $5 \times 10^5$ CFU | 9 | 3 | 12 |
| | | Total | 27 | 9 | 36 |

a. Cohorts will be enrolled as follows: Cohorts 1, 2, and 3 sequentially following a safety review between cohorts.

b. MTBVAC and BCG will be administered intradermally.

## IMMUNOGENICITY ANALYSIS

This section describes collection of raw data from the laboratory, the storage of the raw data, quality control of the raw data, data cleaning, analysis and sharing of the final data with the sponsors in the MTBVAC trial.

**Assays and outcomes:**

- **Whole blood intracellular cytokine assay and flow cytometry (WB-ICS):** Frequencies of antigen-specific CD4+ and CD8+ T cells producing any of 4 cytokines (IL-17, IFN-γ, TNF-α, IL-2), or co-expression patterns of these cytokines simultaneously, following a 12-hour stimulation with MTBVAC. Exploratory

assays will measure responses to stimulation with a peptide pool derived from the amino acid sequence of selected *Mycobacterium tuberculosis* (M.tb) immunodominant epitopes (Lindestam Arlehamn et al., 2016). Cytokine production may also be assessed in additional cell subsets, such as NK, NKT, γδ T cells and MAIT cells, for exploratory analyses. Each assay includes a negative (unstimulated) control and a positive (PHA-stimulated) control, which will be analyzed separately.

- **Whole blood intracellular cytokine assay (WB-ICS) supernatants:** Depending on outcomes of primary analysis and of outcomes of exploratory analysis; future exploratory analysis looking at innate cytokine responses in supernatants harvested after 7 hours of whole blood stimulation with antigens, MTBVAC or a peptide pool of M.tb immunodominant epitopes or controls in the WB-ICS assay or other relevant endpoints may be analyzed.

***Scope of Immunogenicity analysis plan***

The scope of this plan is applicable only to the WB-ICS primary endpoint immunogenicity data collected during the MTBVAC Phase 1b study. It serves as a guide to the personnel involved in generating and analysing the immunology WB-ICS data of the MTBVAC trial.

***Responsibility***

The Postdoctoral Scientist assigned to scientific oversight of immunology for the MTBVAC Phase 1b trial will be responsible for all data management processes outlined in this document. The Postdoctoral Scientist reports to the Deputy Director, Immunology, SATVI and the PI of the vaccine trial.

***Data Flow and procedures***

There are several data generation stages in the MTBVAC trial:

- Follow the appropriate standard operating procedures for WB-ICS sample generation and analysis
- Identification of samples for processing
- Filling and authorisation of sample retrieval form
- Generation of the sample allocation reports

- Completion of the sample processing form
- Sample acquisition on flow cytometer
- Data transfer to the analysis workstation
- Primary data quality control
- Data analysis and cleaning
- Secondary data quality control
- Database lock
- Database sharing with the sponsor

***12hr whole blood intracellular cytokine assay and flow cytometry (WB-ICS)***

***Accuracy, completeness and data quality assurance***

The sample processing forms will be used to crosscheck the raw data to ensure all the samples retrieved from the storage were processed and acquired on the flow cytometer. Any incomplete data will be corrected at this time and discrepancies recorded in the analysis file.

Primary flow cytometry data analysis and quality control (QC) of staining will be performed blinded by a single experienced flow cytometrist. Data from each acquisition file will be loaded onto the FlowJo analysis template as per SATVI standard operating procedures. QC step excludes any technical errors through visual inspection of individual acquired samples according to standard SATVI QC procedures including compensation matrix on beads, antibody staining on cells and acquisition irregularities. This QC step also ensures gates are applied accurately according to predefined gating strategy and compensation as appropriate. The gating strategy will be consistent for all samples in the entire vaccine trial, but gates may be adjusted as needed for each individual participant. Several analysis outcomes may be generated from the analysis template.

Secondly, an experienced, blinded flow cytometrist will check the analyses and QC performed by the first operator (as described below in data quality control) before the data is exported for statistical analysis.

Data will be saved as .csv files and compiled into one master database using R (Version 3.1.2). In addition, R will be used to subtract background (unstimulated samples cytokine response) from stimulated samples

cytokine response. R or Prism will be used for visualization and statistical analysis of absolute frequencies and relative proportions of cytokine-expressing cells (WB-ICS).

***Data quality control***

For all steps listed above, the scientist providing scientific oversight will perform quality control assessment. The quality control checks will involve the following; 1) Manually checking that the values in the compiled master database match the values in individual csv files from 10% of the database or to a maximum of n=10 randomly selected samples; 2) Manually performing background subtraction on these randomly selected samples to ensure background subtraction in R was performed correctly. Errors that may occur in correcting for backgrounds will also be targeted. If an error is identified, corrective action will involve quality control assessments of all the samples and re-analysis of the acquisition files where the error was identified to have come from. A signed form will be provided to certify the quality control checks.

**Acceptability criteria**

The following criteria must be met for samples to be included in the final statistical analysis:

a) Negative (unstimulated) control must be present and interpretable for each set of samples.
b) Frequencies of PHA total cytokine-expressing (WB-ICS) CD4 or CD8 T cells must be greater than the median + 3MAD (median absolute deviation) of the total cytokine CD4 or CD8 T cells of the negative (unstimulated) controls of the entire cohort.
c) For each sample, the frequency of PHA (or positive response to MTBVAC or MegaPool) total cytokine-expressing CD4 T cells must be greater than the frequency of total cytokine-expressing CD4 T cells in the negative (unstimulated) control.

***Database lock***

After final approval of the quality control checks, all files containing the final data will be password-protected, saved on the analysis workstation, and back-ups will be saved and stored on the SATVI backup server under an MTBVAC folder. The final MS Excel, Prism, Pestle, and Spice files will all be placed on the SATVI server upon completion of the clinical trial report.

Should the need for further analysis arise, a duplicate of the master database will be made, named appropriately to indicate reanalysis, and all further analysis done on this new data. The original final analysis will therefore not be amended.

***Database sharing with the sponsor***

Format of the database for sharing with the sponsor will be discussed to best suit both parties. Datasets will be formatted to the agreed requirements; quality controls completed and following authorisation by the SATVI DDI, the data will be sent to external parties.

***Data analysis***

Immunogenicity will be summarized for all time points as collected and as available. No imputation for missing data will be performed. Data will be transformed as appropriate prior to analysis.

For each of the outputs from the relevant immunology parameters, descriptive statistics including means, standard deviations, medians, and interquartile range, will be determined. Between-MTBVAC dose group comparisons of means or medians at each time point, as well as changes between time points post vaccination, will be performed using the most appropriate statistical test.

**Questions to assist R script output:**

1. Is there "vaccine-take" in the three dose groups as determined by antigen specific immune responses at the peak (Day 70) of the immune response compared to that at baseline (Day 7).
    a. Confirm day 70 is the peak by combining all data from all cohorts and look at median of all data from d7, d28, d70 and d180 to determine peak.
    b. Line graphs of individual participants of day 7 versus day 70.
    c. Show data separately for stimulation with MTBVAC or MegaPool.
    d. Analyze separately each dose of MTBVAC as well as groups 1-3 and BCG pooled as a separated group.
    e. Wilcoxon signed rank test.

2. Is there a "memory" response in the three dose groups as determined by antigen specific immune responses at the end of study (Day 180) of the immune response compared to that at baseline (Day 7).
    a. Line graphs of individual participants of day 7 versus day 180 (Stimulation with MTBVAC and Megapool and each group of MTBVAC dose and BCG pooled seperately)
    b. Wilcoxon signed rank test.

3. Is there a difference at peak or end of study when vaccinating with increasing doses of MTBVAC in comparison to BCG?

    a. Present both 1 & 2 above as Box and whisker plots of day 70 and day 180 of all 4 groups split by MTBVAC or MegaPool stim.
    b. Create two tables to display Kruskal-Wallis followed by Mann-whitney and FDR for the 12 comparisons.

| Comparison of MTBVAC by escalating dose | | | | | |
|---|---|---|---|---|---|
| Kruskal-Wallis | | | | | |
| | Day 70 | | Day 180 | | FDR of ... indicates significance shown by highlighting appropriate Mann-Whitney p-values in bold and red. |
| | MTBVAC | MegaPool | MTBVAC | MegaPool | |
| Cohort 1 versus Cohort 2 | | | | | |
| Cohort 1 versus Cohort 3 | | | | | |
| Cohort 2 versus Cohort 3 | | | | | |

| Comparison of dose-escalation of MTBVAC to BCG (standard dose) | | | | | |
|---|---|---|---|---|---|
| Kruskal-Wallis | | | | | |
| | Day 70 | | Day 180 | | FDR of ... indicates significance shown by highlighting appropriate Mann-Whitney p-values in bold and red. |
| | MTBVAC | MegaPool | MTBVAC | MegaPool | |
| Cohort 1 versus BCG | | | | | |
| Cohort 1 versus BCG | | | | | |
| Cohort 2 versus BCG | | | | | |

4. What is the quality of the immune response?
    a. Split the immune responses by stimulation and into CD4 and CD8 T cells and MTBVAC and MegaPool and by standard boolean responses grouped over time. No statistical analysis.

**Reference:**

Lindestam Arlehamn, C. S., McKinney, D. M., Carpenter, C., Paul, S., Rozot, V., Makgotlho, E., et al. (2016). A Quantitative Analysis of Complexity of Human Pathogen-Specific CD4 T Cell Responses in Healthy M. tuberculosis Infected South Africans. *PLoS Pathog*, *12*(7), e1005760. http://doi.org/10.1371/journal.ppat.1005760